Carcinogenesis vol.13 no.3 pp.315–319, 1992

Identification of N^2-substituted 2′-deoxyguanosine-3′-phosphate adducts detected by ^{32}P-postlabeling of styrene-oxide-treated DNA

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Styrene-7,8-oxide, a metabolite of the industrial chemical styrene, was reacted with calf thymus DNA. Six adducts were detected by ^{32}P-postlabeling. The two diastereomers of N^2-(2-hydroxy-1-phenylethyl)-2′-deoxyguanosine-3′-phosphate and the corresponding *N*-1 substituted compounds were isolated from the aqueous reaction mixture of 2′-deoxyguanosine-3′-phosphate and styrene-7,8-oxide (pH 10.5) and characterized by liquid secondary-ion and four-sector tandem mass spectrometry, ultraviolet, circular dichroism, and fluorescence spectrophotometry, and ^{32}P-postlabeling. Co-chromatography of the DNA–styrene-7,8-oxide reaction products with the synthetic standards showed that adduct no. 6 arose as a result of aralkylation at the N^2-exocyclic site of the guanine base. The recovery of the N^2-adduct was dependent on the concentration of the solvent used during octadecylsilyl chromatography. These studies revealed that the N^2-guanosine derivatives are the major products of the reaction of DNA and styrene-7,8-oxide *in vitro* detected by ^{32}P-postlabeling.

Introduction

Styrene, a bulk plastics chemical, is metabolized by the P450 enzyme system to styrene-7,8-oxide, which has been shown to be mutagenic (1–3) and carcinogenic (4,5). Covalent adducts of styrene-7,8-oxide with nucleosides and DNA have been found (6,7). The formation of styrene-7,8-oxide–DNA adducts may be responsible for the genotoxic effects of styrene.

In previous studies we have detected styrene-7,8-oxide–DNA adducts by ^{32}P-postlabeling (8); modifications at the guanine base accounted for the majority of the adducts. These adducts can be identified by ^{32}P-postlabeling and chromatography of synthetic modified 3′-phosphorylated nucleotides of known structure, which are substrates for T4 polynucleotide kinase. Comparison chromatography of these standards with the DNA reaction products can be used to assign structures to the unknown DNA adducts. Two chromatography spots were identified by the synthesis of O^6-(2-hydroxy-2-phenylethyl)-2′-deoxyguanosine-3′-phosphate (β isomer) and the corresponding α isomer (9). As established by co-chromatography, these two compounds appeared on the autoradiograms of the products from the reaction of styrene-7,8-oxide with DNA as adducts no. 4 and no. 5 (9). Because the *N*-7 alkylated 2′-deoxyguanosine derivatives may undergo either depurination or imidazole ring opening (10,11), their detection requries modification of the postlabeling procedure (12,13). The N^2-derivatives, however, like the O^6-alkyl compounds, are very stable and presumably would appear on the adduct map. High yields of N^2-substituted styrene-7,8-oxide-guanosine derivatives have been obtained under alkaline conditions *in vitro* (14). In these studies, we sought to determine which of the detected adducts on the ^{32}P-postlabeling adduct map correspond to the N^2-substituted compounds.

*Abbreviations: LSIMS, liquid secondary-ion mass spectra; MS/MS, tandem mass spectrometry; CID, collision-induced dissociation; CD, circular dichroism; 3′-dGp, 2′-deoxyguanosine-3′-phosphate; ODS, octadecylsilyl.

Materials and methods

Experimental

TLC was performed on polyethyleneimine (PEI) ion-exchange plates (Polygram Cel 300 PEI/UV 254) and on Whatman KC18 reversed-phase plates. UV spectra were recorded on a Beckman DU-7 spectrophotometer. Liquid secondary-ion mass spectra (LSIMS*) were obtained in the negative-ion mode with a Kratos MS-50S mass spectrometer (Manchester, UK) equipped with a 23 kg magnet and post-accelerator detector. A Cs^+ primary ion beam of 10 keV was used. Tandem mass spectrometry (MS/MS) was performed in the positive-ion mode on a Kratos Concept II HH four-sector instrument of EBEB geometry. The sample was ionized as above in a LSIMS source with a 12 keV Cs^+ beam. Only the ^{12}C isotope peak for the MH^+ ion cluster was selected in MS-I and introduced into a collision cell containing helium. The fragment ions produced by collision-induced dissociation (CID) were separated in MS-II and detected with an optically coupled, 1000 channel array detector. Circular dichroism (CD) spectra were recorded on an upgraded G-5 spectropolarimeter (Japan Spectroscopy Co. Ltd, Tokyo, Japan), which was coupled with a personal computer for data collection and storage.

Starting materials and reagents were purchased from Sigma Chemical Co. (St Louis, MO), Aldrich Chemical Co., Inc. (Milwaukee, WI) and Fisher Scientific Co. (Pittsburgh, PA); enzymes from Worthington Biochemical Corporation (Freehold, NJ), Bethesda Research Laboratories (Gaithersburgh, MD) and Sigma; radiolabeled compounds from Amersham (Arlington Heights, IL). HPLC was performed using a Perkin-Elmer system equipped with an ISCO gradient programmer and Econosil C-18 columns (Alltech 10 μ 10 × 250 mm, 4.6 × 250 mm).

Synthesis of N-1- and N^2-(2-hydroxy-1-phenylethyl)-2′-deoxyguanosine-3′-phosphate 1aI, 1aII, 2aI, 2aII

Twenty milligrams of 2′-deoxyguanosine-3′-phosphate (3′-dGp) was reacted overnight with 200 μl of styrene oxide in 4 ml of ammonium carbonate buffer (pH 10.5, 20 mM) at 37°C. The reaction mixture (adjusted to pH 7) was applied to a previously equilibrated DEAE Sephadex column (2.5 cm × 20 cm, 0.02 M ammonium acetate, pH 7). Four milliliter fractions were collected, and the UV absorbance was monitored at 254 nm. The products were eluted with a gradient of 1 M ammonium acetate, pH 7. Fractions 56–62 (1a) and 63–73 (2a) were pooled and lyophilized. These crude products were further purified by HPLC (0–70% methanol in 10 mM ammonium acetate, pH 5.1, in 60 min, and to 100% methanol in 10 min at a flow rate of 3 ml/min). 1aI (t_R 32 min), 1aII (t_R 36 min) LSIMS *m/z* 466 $[M-H]^-$, UV_{max} (pH 6) 255 nm, UV_{max} (pH 1) 259 nm, UV_{max} (pH 12) 256 nm. 1aI and 1aII were depurinated in 1 N HCl at 100°C for 3 h and purified by HPLC. 1bI (t_R 46 min), 1bII (t_R 49 min) UV_{max} (pH 6) 248 nm, 277 nm, UV_{max} (pH 1) 251 nm, UV_{max} (pH 12) 278 nm.

2aI (*R* enantiomer) (t_R 24 min), 2aII (*S* enantiomer) (t_R 27 min) LSIMS *m/z* $[M-H]^-$ 466 UV_{max} (pH 6) 255 nm, UV_{max} (pH 1) 260 nm, UV_{max} (pH 12) 260 nm. 2aI and 2aII were depurinated as described above. 2bI (t_R 40 min), 2bII (t_R 43 min) UV_{max} (pH 6) 250, 274 nm; UV_{max} (pH 1) 252 nm; UV_{max} (pH 12) 276 nm. LSIMS *m/z* MH^+ 272.

In vitro reaction

One-half milligram of purified calf thymus DNA was dissolved in 0.5 ml of 10 mM Tris–HCl buffer, pH 7.4, and was reacted with 5 μl of styrene oxide. The mixture was incubated overnight at 37°C. Unreacted styrene oxide was removed by diethyl ether extraction.

^{32}P-postabeling

^{32}P-postlabeling of the synthesized standards and the DNA–styrene-oxide reaction products was performed as described earlier (8,9). The ^{32}P-postlabeled

reaction mixtures were applied to a 10 × 10 cm octadecylsilyl (ODS) plate with a paper wick attached and developed overnight with 0.4–2.0 M ammonium formate, pH 6.2. In several experiments, autoradiography was performed on the ODS plates to locate the adducts before contact transfer to the PEI plates. DNA adducts of the ODS chips were contact transferred to the PEI–cellulose sheets as previously described (8,9). The adducts on the PEI plates were separated chromatographically as previously described (9) using the following solvent systems: 1D, 1.8 M lithium formate, 4.25 M urea (pH 3.5); 2D, 0.22 M Tris–HCl, 0.36 M lithium chloride, 3.8 M urea (pH 8.0); and 3D, 1.7 M sodium phosphate (pH 6.0). After chromatography, the plates were washed with water and air dried. The adducts were located by autoradiography using Kodak XAR-5 film and an intensifying screen (Cronex Lightning Plus, Du Pont, Boston, MA). The radioactive spots on the PEI sheets detected by autoradiography were scraped into liquid scintillation vials containing 5 ml of scintillation cocktail (Safety-Solve, Research Products, Inc., Mount Prospect, IL) and counted in a liquid scintillation counter. Regions adjacent to the radioactive spots were also scraped into scintillation vials and counted to determine the background radioactivity.

Results

Using ^{32}P-postlabeling, we detected six adducts from the reaction of DNA with styrene-7,8-oxide *in vitro*. Adducts 1 and 2 together accounted for 83.2% of the total; adducts 3, 4, 5 and 6 accounted for 8.8, 1.7, 1.1 and 5.1% respectively (9). To determine which of these adducts corresponded to the N^2-substituted derivatives, chromatography standards were prepared by reacting styrene-7,8-oxide and 3′-dGp under alkaline conditions. DEAE–Sephadex ion-exchange chromatography followed by HPLC separation isolated four major components (1aI, 1aII, 2aI, 2aII). LSIMS mass spectra recorded in the negative-ion mode for 1aI, 1aII, 2aI and 2aII were identical. The spectra showed a molecular ion $[M-H]^-$ at *m/z* 466 in each case, identifying a mol. wt of 467. These spectra are consistent with the addition of a hydroxyphenylethyl group to 3′-dGp. The UV spectra of 1aI and 1aII over a pH range of 1–12 were identical (Figure 1), and analysis of the spectra showed that both fractions are *N*-1-substituted derivatives (15). UV spectra of 1bI and 1bII, the depurinated bases of 1aI and 1aII, confirmed the *N*-1 substitutions. The UV spectra of 2aI and 2aII (Figure 2) suggested that they are N^2-substituted compounds (14). Both the *N*-1 and N^2-derivatives were fluorescent and their emission spectra were dependent on pH. 2aI and 2aII, the N^2-styrene oxide adducts, were fluorescent under alkaline pH (data not shown), as has been previously described for other N^2-substituted guanosine derivatives (16). In contrast, the *N*-1 derivatives were fluorescent under acidic conditions (Figure 3).

The depurinated bases obtained from 2aI and 2aII were further studied by high-energy CID tandem MS experiments. The CID mass spectrum of 2bI with MH^+ at *m/z* 272 is shown in Figure 4. 2bII exhibited the same CID mass spectrum (not shown). The MH^+ ion at *m/z* 272 corresponds to guanine containing one hydroxyphenylethyl group. The ions at *m/z* 254 and 240 correspond to loss of H_2O and $-CH_2OH$ respectively from MH^+, which suggests α substitution in the parent compound. Additional fragments at *m/z* 152 and 135 were identified as guanine and guanine-NH_3 respectively. Ions at *m/z* 121 and 103

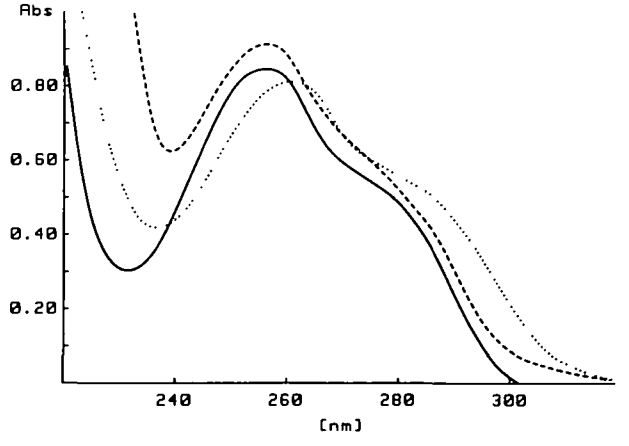

Fig. 1. UV spectra of *N*-1-(2-hydroxy-1-phenylethyl)-2′-deoxyguanosine-3′-phosphate, 1aI. Dotted line, pH 1; solid line, pH 6; dashed line, pH 12.

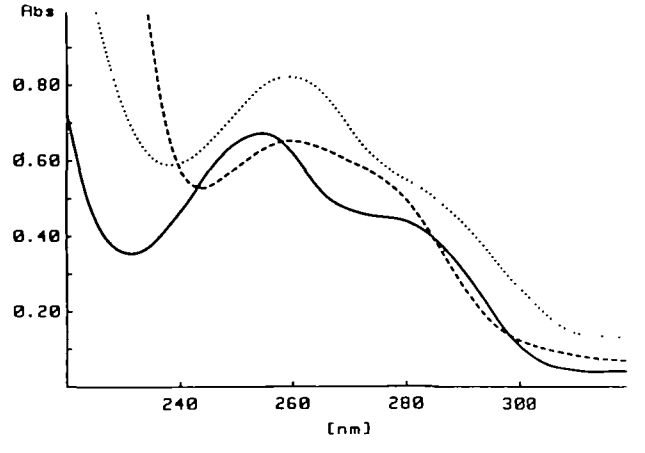

Fig. 2. UV spectra of N^2-(2-hydroxy-1-phenylethyl)-2′-deoxyguanosine-3′-phosphate, 2aI. Dotted line, pH 1; solid line, pH 6; dashed line, pH 12.

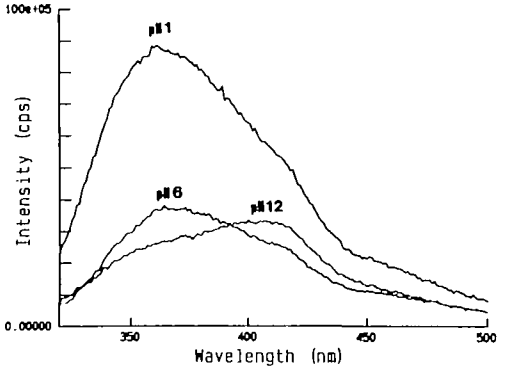

Fig. 3. Fluorescent emission spectra of *N*-1-(2-hydroxy-1-phenylethyl)-2′-deoxyguanosine-3′-phosphate, 1aI.

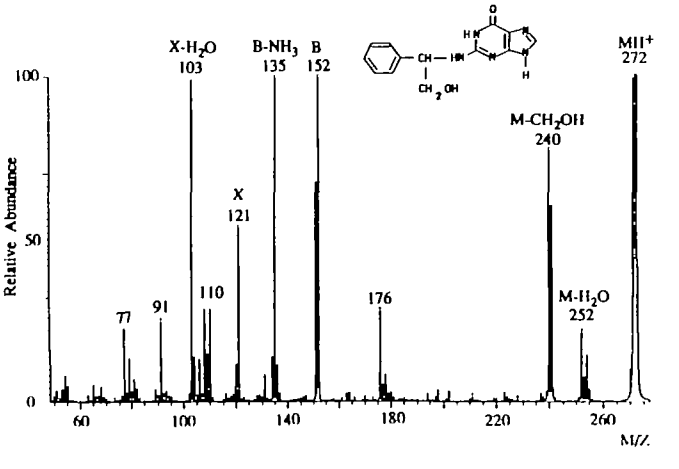

Fig. 4. High-energy CID tandem mass spectrum of 2bI. B = guanine + H: *X* = 2-hydroxy-1-phenylethyl-.

represent the hydroxyphenylethyl moiety and the same moiety with the loss of 1 mol of water respectively. The fragment at *m/z* 176 has not yet been identified. The results of these mass spectrometry studies indicate that the structures of both 2aI and 2aII are N^2-(2-hydroxy-1-phenylethyl)2′-deoxyguanosine-3′-phosphate.

Comparison of the CD spectra of 2aI and 2aII recorded in water with the published CD spectra of diastereomeric N^2-(2-hydroxy-1-phenylethyl)-guanosines (14) showed that 2aI was the *R* enantiomer and 2aII was the *S* enantiomer (Figure 5).

Figure 6(A) shows the autoradiogram of ^{32}P-postlabeled 2aI. 2aII showed the same mobility upon ^{32}P-postlabeling (not shown). Figure 6(B) is the autoradiogram obtained after co-chromatography of ^{32}P-postlabeled 2aII with ^{32}P-postlabeled DNA–styrene-7,8-oxide reaction products on PEI plates. Comparison of these two autoradiograms shows identical mobility of ^{32}P-postlabeled 2aII and adduct no. 6 from the DNA–styrene-7,8-oxide reaction. This suggests that adduct no. 6 corresponds to the N^2-substituted adducts. Confirmation of this result was made by enzymatic digestion of styrene-7,8-oxide-modified DNA. The resulting 3′-dNps were separated by HPLC and the fractions corresponding to the elution time of the N^2-adducts were collected and ^{32}P-postlabeled. The adducts detected by postlabeling corresponded to adduct no. 6.

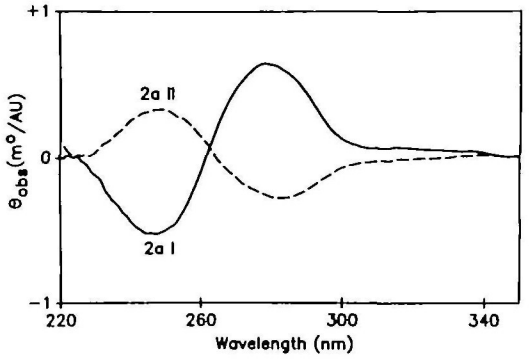

Fig. 5. CD spectra of N^2-(2-hydroxy-1-phenylethyl)-2′-deoxyguanosine-3′-phosphate, 2aI and 2aII.

Our results indicated that the N^2-adduct detected by post-labeling (adduct no. 6) was a minor component of the DNA–styrene-7,8-oxide products (8,9). The low level of the N^2-adduct suggested a substantial loss of this product during either postlabeling or during chromatography. The labeling efficiency of N^2-(2-hydroxy-1-phenylethyl)-2′-deoxyguanosine-3′-phosphate was compared to that of 3′-dGp. Each compound (0.4 ng) was ^{32}P-postlabeled and chromatographed on PEI plates with 1.5 M ammonium formate, pH 3.5. Comparison of the radioactivity in the N^2-adduct with that of 3′-dGp indicated the labeling efficiency to be 84%. No difference in labeling efficiency was observed with the two diastereomers. We therefore investigated the retention of the styrene-7,8-oxide–DNA adducts during ODS chromatography of the ^{32}P-postlabeled DNA–styrene-7,8-oxide reaction mixture with developing solvents of different molarities. Figure 7 shows the autoradiograms of the ODS plates developed overnight in 0.4, 1.0 and 2.0 M ammonium formate, pH 6.2.

In the ODS plate eluted with 0.4 M ammonium formate (Figure 7A), a single radioactive area remains at the origin. In contrast to these results, the ODS plates eluted with ammonium formate, either 1.0 M (Figure 7B) or 2.0 M (Figure 7C), show two additional ^{32}P-postlabeled areas. Comparison of the plates developed in 1.0 and 2.0 M solvent shows that the distance of migration of these radioactive areas is inversely related to the solvent concentration. The ODS chips containing radioactive areas labeled as (a), (b) and (c) were transferred to PEI plates and further chromatographed. For the ODS plates eluted with 0.4 M ammonium formate, only area (a) showed detectable adducts on subsequent chromatography. The adduct distribution was as previously observed (9). PEI chromatography of area (a) of the ODS plate eluted with 1.0 M ammonium formate gave the same product distribution as observed for area (a) of the ODS plate eluted with 0.4 M ammonium formate (Figure 8A). PEI chromatography of areas (b) and (c) resulted in the detection of a single adduct, no. 6 (Figure 8B and C). Results similar to those obtained with 1.0 M ammonium formate were also obtained with PEI chromatography of areas (a), (b) and (c) of the ODS plate eluted with 2.0 M ammonium formate.

The percentage of the total modification detected for each

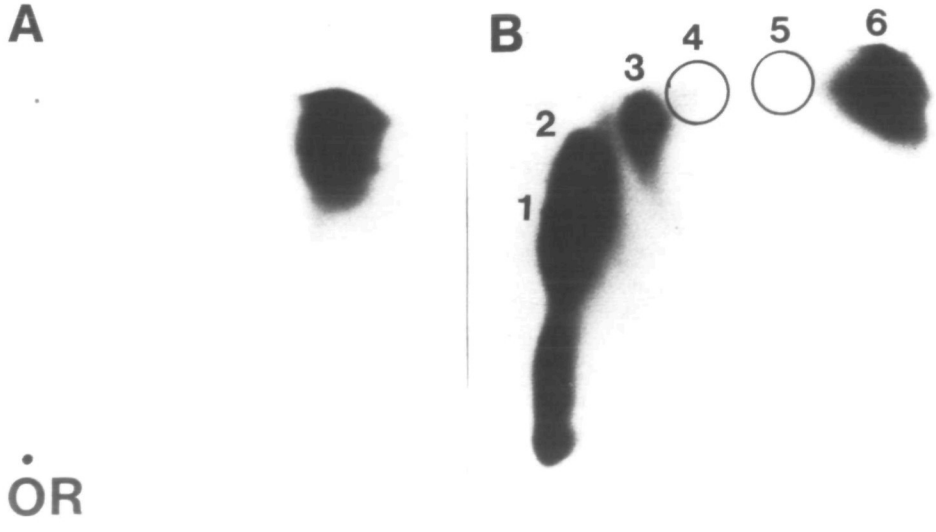

Fig. 6. (A) Autoradiogram of ^{32}P-postlabeled 2aI. The film was exposed for 15 min at room temperature. (B) Autoradiogram of ^{32}P-postlabeled DNA–styrene-oxide reaction mixture co-chromatographed with 2aII. The film was exposed for 1 h at room temperature.

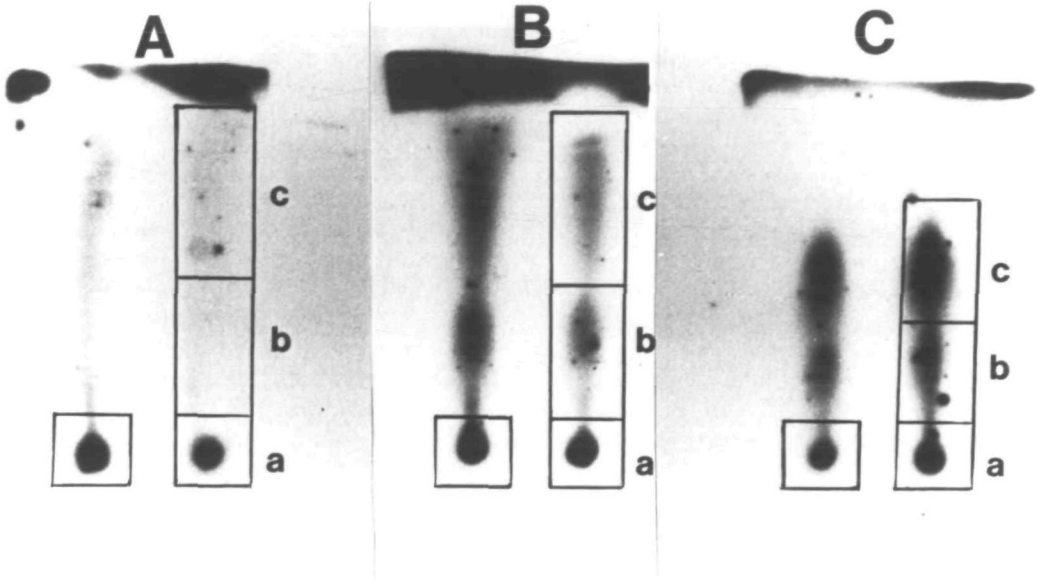

Fig. 7. Autoradiograms of the ODS plates developed overnight in 0.4 M (**A**), 1.0 M (**B**) and 2.0 M (**C**) ammonium formate, pH 6.2. DNA–styrene-oxide reaction mixtures were spotted at the origin. The film was exposed for 5 min at room temperature.

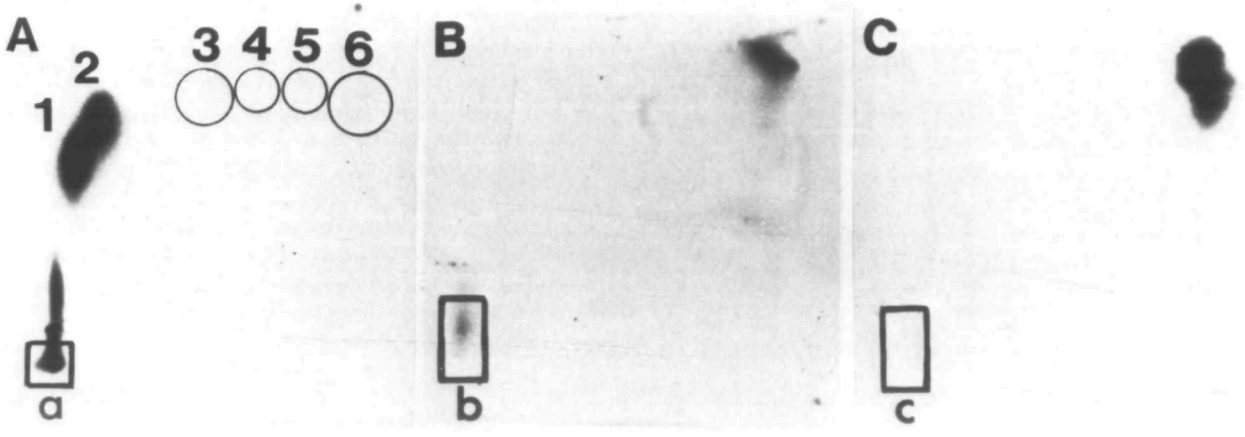

Fig. 8. Autoradiograms obtained after PEI ion-exchange chromatography of the transferred ODS chips a, b and c, respectively, from the ODS plate developed in 1.0 M ammonium formate, pH 6.2 (Figure 7B). The film was exposed for 1 h at room temperature.

adduct after summing the radioactivity detected by PEI chromatography of areas (a), (b) and (c) from the ODS plate is shown in Table I. Comparison of the relative percentage of the six adducts detected shows that as the molarity of ammonium formate increases from 0.4 to 2.0 M, the level of adduct no. 6 increases from 17% of the total to 73%. Conversely, the percentage of the total of adducts 1–3 decreases as the solvent molarity increases. The levels of adducts 4 and 5 remain approximately the same. In addition the relative adduct level increased from 3.5×10^{-6} for 0.4 M ammonium formate to 1.21×10^{-5} and 1.64×10^{-5} for 1.0 and 2.0 M ammonium formate respectively. Recovery of the N^2-adduct from the ODS plate was determined. N^2-(2-Hydroxy-1-phenylethyl)-2′-deoxy-guanosine-3-′-phosphate (0.4 ng) was ^{32}P-postlabeled and chromatographed on PEI–cellulose either with or without purification by chromatography on an ODS plate eluted with 2.0 M ammonium formate. From this comparison the recovery of the N^2-adduct from the ODS plate was estimated to be 83%.

Table I. Distribution of DNA–styrene-oxide reaction products detected by ^{32}P-postlabeling using different buffer concentrations in the ODS chromatography step

Adduct	Ammonium formate (pH 6.2)		
	0.4 M	1.0 M	2.0 M
1 and 2	72.0%	40.6%	23.8%
3	8.9%	2.7%	2.2%
4 and 5	1.2%	1.3%	0.7%
6	17.7%	55.3%	73.2%

Discussion

Previous investigations have shown that reaction of guanosine and deoxyguanosine with styrene-7,8-oxide at neutral pH leads to the formation of *N*-7-substituted guanine adducts (6). In contrast, under alkaline conditions, the *N*-1 and N^2 positions of guanine are the most reactive. Therefore, to obtain N^2-substi-

tuted 3′-dGp derivatives, we reacted 3′-dGp with styrene-7,8-oxide at pH 10.5 and isolated four products. Based on their UV and mass spectra they were identified as the *N*-1 and N^2-styrene-7,8-oxide substituted 3′-dGp derivatives. Nucleophilic opening of the epoxide ring can occur through either the α or β positions. Mass spectrometry analysis indicated that the N^2-derivatives were formed through ring opening at the α position, which is consistent with earlier studies (17). Since this α carbon is chiral, two enantiomers are possible. Circular dichroic analysis of our products allowed determination of the *R* and *S* enantiomers of the N^2-derivatives. After ^{32}P-postlabeling, the two enantiomers, 2aI and 2aII, showed different mobilities on the ODS plates (R_f 2aI > R_f 2aII; data not shown), but were not resolved under the chromatographic condition(s) used on the PEI plates.

Comparison of the chromatographic properties of ^{32}P-postlabeled N^2-substituted 3′-dGp and ^{32}P-postlabeled DNA reacted with styrene-7,8-oxide allowed identification of adduct 6 as corresponding to N^2-substituted 3′-dGp. Previously, we have shown that adducts 4 and 5 correspond to O^6-substituted 3′-dGp adducts. As detected by ^{32}P-postlabeling, the ratios of formation of the N^2-derivatives and the O^6-derivatives were approximately 2:1 (9). These results were in contrast to those of Vodicka and Hemminki (15), which indicated that the N^2-products were formed to a greater extent in DNA reacted with styrene oxide compared with the O^6-derivatives. This suggested that we were losing some of the N^2-derivatives. We investigated the labeling of the N^2-adducts by T4 polynucleotide kinase and found it to be ~84%. This is in general agreement with the high labeling efficiency for other aromatic N^2-adducts (18,19).

The chromatographic retention of the N^2-derivatives on the ODS plates was highly dependent on the concentration of the buffer. At low ionic strength, most of the N^2-derivative was not retained on the ODS plate. As the ionic strength increased, the N^2-derivatives were increasingly retained on the plate, but not exclusively at the origin. PEI chromatography of area (a) gave the same adduct map as previously reported (9). PEI chromatographic analysis of areas (b) and (c) of the ODS plate resulted in adducts corresponding primarily to N^2-compounds. Quantitative analysis of the ODS plates eluted with 1–2 M ammonium formate showed that the N^2-adducts are the principal adducts detected.

The recovery of adducts 1, 2 and 3, in contrast to adduct 6, was not influenced by the solvent used to elute the ODS plate. These adducts appear to be quantitatively retained at the origin. Adducts 1 and 2 have been identified using mass spectrometry as bis(2-hydroxy-2(1)-phenylethyl)-2′-deoxyguanosine-3′,5′-bisphosphate derivatives (S.Kaur, manuscript in preparation). Studies are in progress to identify further these structures.

The long-term goal of our studies is to use ^{32}P-postlabeling to analyze for the presence of DNA adducts in the lymphocytes of workers occupationally exposed to styrene. Analysis of these results can be confounded by the presence of endogenous adducts (20,21), and smoking- (22,23) and diet-related DNA adducts (24). Identification of styrene-7,8-oxide–DNA adducts detected by ^{32}P-postlabeling should allow us to determine which of the adducts detected in peripheral blood mononuclear cells are related to styrene exposure.

Acknowledgements

We thank Stephen Ordway for editorial assistance. Supported by the Health Effects Component of the University of California Toxic Substances Research and Training Program, NIEHS grant P42ES04705, NIH grant RR01614, NSF grant DIR 8700766, and NIOSH grant RO1 0H02221.

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Received on March 15, 1991; revised on November 15, 1991; accepted on December 3, 1991